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Maternal sucralose intake alters gut microbiota of offspring and exacerbates hepatic steatosis in adulthood

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ABSTRACT

Background: Nonalcoholic fatty liver disease (NAFLD) is considered to be associated with diet and gut dysbiosis. Excessive sucralose can induce gut dysbiosis and negatively affect host health. Maternal diet shapes the microbial communities of neonate and this effect continues in later life. We aimed to investigate the effects of maternal sucralose (MS) intake on the susceptibility of offspring to hepatic steatosis in adulthood.

Methods: C57BL/6 pregnant mice were randomized into MS group (MS during gestation and lactation) and maternal control (MC) group (MC diet). After weaning, all offspring were fed a control diet until 8 weeks of age, and then treated with a high-fat diet (HFD) for 4 weeks. The intestinal development, mucosal barrier function, and gut microbiota were assessed in the 3-week-old offspring. Moreover, the severity of hepatic steatosis, serum biochemistry, lipid metabolism, and gut microbiota was then assessed in the 12th week.

Results: MS significantly inhibited intestinal development and disrupted barrier function in 3-week-old offspring. MS also induced intestinal low-grade inflammation, significantly changed the compositions and diversity of gut microbiota including reducing butyrate-producing bacteria and cecal butyrate production with down-regulation of GPR43. Mechanically, blocking GPR43 blunted the anti-inflammatory effect of one of the butyrate-producing bacteria, *Clostridium butyricum in vitro*. After HFD treatment, MS exacerbated hepatic steatosis, and disturbed fatty acid biosynthesis and metabolism, accompanied by inducing gut dysbiosis compared with MC group.

Conclusions:MS intake inhibits intestinal development, induces gut dysbiosis in offspring through down-regulation of GPR43, and exacerbates HFD-induced hepatic steatosis in adulthood.

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Introduction

As one of the most prevalent chronic liver diseases throughout the world, nonalcoholic fatty liver disease (NAFLD) is considered to be connected with inappropriate nutrition. In recent years, increasing pieces of evidence prove that gut microbiota has appeared as a critical environmental aspect in the development of NAFLD.¹ Given that the perinatal period is a pivotal developmental stage for the neonatal microbiome,² it is widely believed that the diet of pregnant and lactating mothers can shape the microbial communities of the neonate at birth and this effect continues in later life.³ Interestingly, Umesh D. Wankhade et al.

reported that maternal high-fat diet (HFD) could induce gut dysbiosis of offspring and enhance their predisposition to hepatic steatohepatitis.⁴

Sucralose, an organochlorine artificial sweetener, has been widely used around the world. It is increasingly added into commonly consumed foods such as soft drinks, cereals, and sugar-free desserts. However, accumulating evidence shows that sucralose can alter the composition of the gut microbiome, affect host health, and induce hepatic diseases.^{5–8} Bian X et al. showed that sucralose consumption elevated pro-inflammatory gene expression in the liver by disrupting the gut

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microbiota in mice. Dhurandhar D et al. revealed that an obvious hepatic damage appeared on daily ingestion of sucralose in rats. Chih-Wei Liu et al. found that liver protein metabolism disorder appeared in sucralose-treated mice. More importantly, maternal exposure to sucralose can downregulate progeny's hepatic detoxification and alter progeny's gut microbiome. Considering the potential influence of maternal sucralose (MS) administration on the liver and the negative effect in offspring on gut microbiota, we assume that MS can cause gut microbiota dysbiosis, thereby increasing hepatic steatosis susceptibility when the offspring reach adulthood.

In our present study, we found that MS inhibited intestinal development, induced imbalance of gut microbiota and low-grade inflammation, and further disrupted gut barrier function in offspring. The butyrate-producing bacteria and butyrate were dramatically decreased by MS. And finally, MS exacerbated HFD-induced hepatic steatosis in adulthood. From *in vitro* experiments we found that *Clostridium butyricum* decreased inflammatory cytokines production through activating GPR43. These data suggest that excessive sucralose should be taken with caution especially during pregnancy and lactation. Thus, it provides new insight into a better understanding of the pathogenesis of NAFLD in adulthood.

Results

Subject characteristics of offspring mice

In this study, pups' sex ratio and overall litter sizes were not significantly different between MS and MC groups. No death was found in the two groups. The body weight of two groups was similar at other measured time points before 8 weeks except that the offspring in MS group were heavier than those in MC group at 2–4 weeks of age. During the period with HFD, although no difference was found in the body weight at the very beginning, the offspring in MS group were distinctly heavier than those in MC group after 15 days with HFD. Thus, MS supplementation slightly increased weight gain in offspring at weaning and significantly aggravated weight gain to HFD (Figure 1b).

MS inhibited intestinal development and disrupted gut barrier in 3-week-old offspring

The development of the gastrointestinal tract is mainly influenced by maternal nutrition until the third week after birth in mice. 13,14 So we investigated the effect of sucralose on the intestinal development and integrity of gut barrier in 3-week-old offspring. We measured the villi length and crypt depth of 3-week-old offspring. The length and depth of 3-week-old pups in MS group were significantly shorter than those in MC group (Figure1c-d). Gut barrier, in physical, consists of mucous layer, intestinal epithelial cells, as well as the tight junctions located at the top of the intestinal membrane. 15 Epithelial cells renew by cell proliferation, differentiation, and migration. The number of Ki67 positive proliferating cells per crypt was counted, and decreased Ki67 positive cells were observed in MS group compared with MC group (Figure 2a). Similarly, the number of Periodic Acid Schiff (PAS) positive cells (indicating goblet cells) also decreased (Figure 2b). MUC2, one of the most important products of goblet cells, is closely related to the formation of mucous layer. According to the number of MUC2 positive cells and the mRNA expression, MUC2 production was downregulated in MS group compared with MC group (Figure 2c–d).

The tight junction proteins including ZO-1, Claudin-1, Claudin-3, and Occludin are considered as the most important factors in determining the integrity of gut barrier. 16 According to polymerase chain reaction (PCR) data, the expression of Claudin-1, Claudin-3, and ZO-1 significantly decreased in MS group, while that of Occludin was similar in the two groups (Figure 3a). Besides, immunofluorescence also suggested that the expression of ZO-1 protein decreased in MS group (Figure 3b). Immunoglobulin A (IgA), functioning as inhibiting pathogens from adhering to the mucous, plays a critical role in the gut immunological barrier function.¹⁵ As immunofluorescence of IgA showed, MS dramatically decreased the level of IgA expression in the small intestine (Figure 3c-d). These data indicate that MS can inhibit intestinal development and disrupt the integrity of gut barrier in early life of offspring.

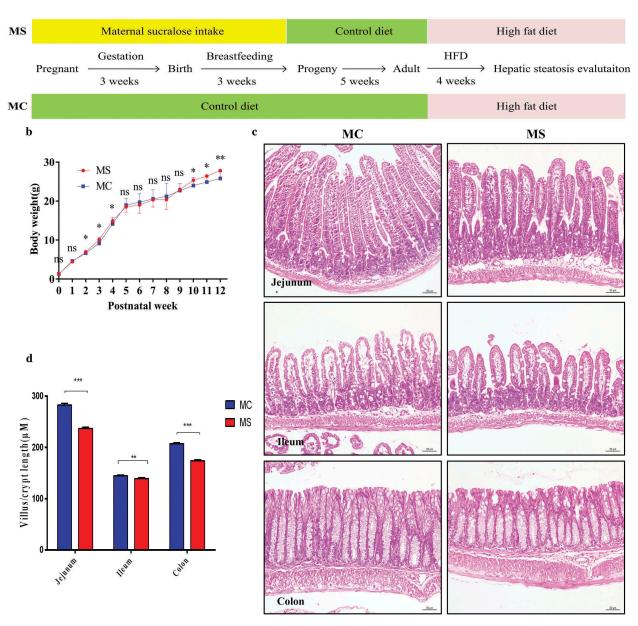


Figure 1. Maternal sucralose intake inhibited the intestinal development in 3-week-old pups. The experiment protocol was described in (a). Bodyweight of pups was recorded weekly up to 12 weeks (b). Small intestinal and colonic tissues of 3-week-old mice were detected by H&E staining (c). And villi length and crypt depth of at least 100 villi and crypts/mice were detected (d).MC, maternal control diet. MS, maternal sucralose intake. HFD, high-fat diet.MC: n = 19, MS: n = 17. Scale bar: 50 μ m. *p < .05, **p < .01, ***p < .001.

MS-induced intestinal low-grade inflammation in 3-week-old offspring

a

In this study, we have not found apparent microscopic inflammation in either small intestine or colon by H&E staining in two groups (Figure 1c). We further assessed the levels of the proinflammatory cytokine including IL-1 β , IFN- γ , and TNF- α

in colon tissues. Interestingly, the relative mRNA expression levels of these proinflammatory cytokines were significantly higher in MS group than those in MC group (Figure 2e). These results implied that MS supplementation could induce intestinal low-grade inflammation and impair gut barrier function in offspring.

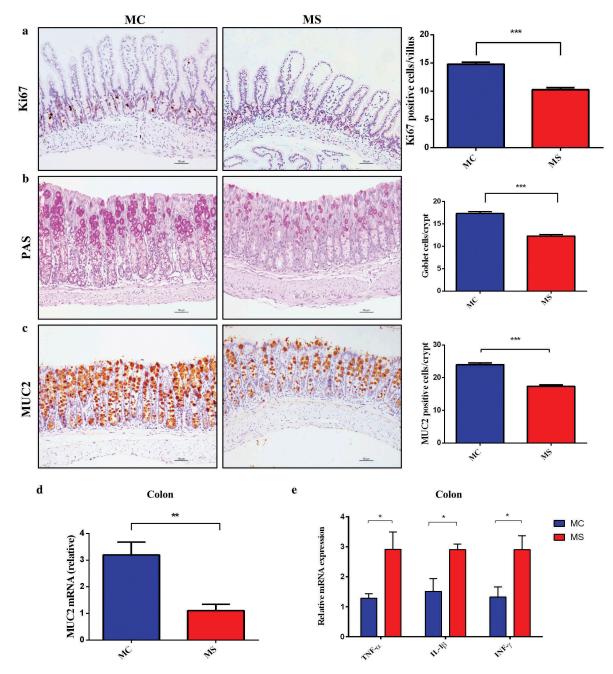


Figure 2. Maternal sucralose intake disturbed intestinal proliferation and differentiation and induced colonic low-grade inflammation of 3-week-old pups. Ki67 staining in the small intestine (a). Periodic acid Schiff staining for Goblet cells and MUC2 staining in the colon was shown. The number of positive staining cells in each crypt was also measured (b–c). Total RNA was extracted from the colonic tissues for Real-time-PCR analysis. The relative expression of MUC2 was presented (d). The relative expression of inflammatory cytokines including TNF-α, IL-1β, and IFN-γ was shown (e). MC, maternal control diet.MS, maternal sucralose intake. MC: n = 7, MS: n = 5. Scale bar: 50 μm. **p < .01, **p < .01, ***p < .001.

MS altered the composition of gut microbiota in 3-week-old offspring mice

The gut microbiota of 3-week-old offspring was characterized and quantitatively analyzed via 16 S rRNA sequencing. We assessed operational taxonomic units(OTUs) of MS group and MC group

to identify the shared and unique species. Venn diagram showed that there were 356 OTUs in MC group and 281OTUs in MS group, with 268 OTUs shared (Figure 4a). Then, we analyzed the microbiota community structure in two groups. There were four dominant bacteria at phylum level, which were *Firmicutes*, *Bacteroidetes*,

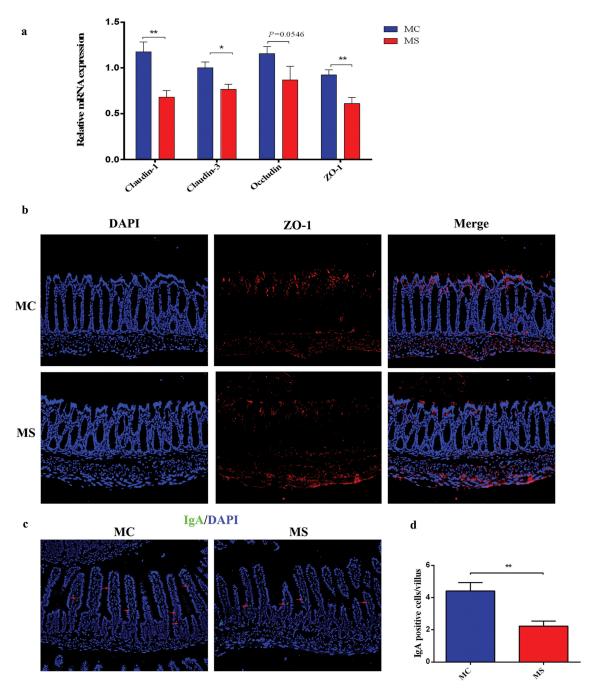


Figure 3. Maternal sucralose intake destroyed tight junction in the colon and decreased IgA in the small intestine of 3-week-old pups. The relative expression of ZO-1, Claudin-1, Claudin-3, and Occludin was shown (a). Detection of ZO-1membrane location by immunostaining (red, 200×), DAPI (blue) counterstains nuclei (b). IgA in the small intestine was presented by immunostaining analysis (green, 200x), and DAPI (blue). IgA positive cells were pointed by arrows (c). IgA positive cells in 100 villi were counted (d). MC, maternal control diet. MS, maternal sucralose intake.MC: n = 7, MS: n = 5. *p < .05, **p < .01.

Proteob-acteria, and Verrucomicrobia. The relative abundance of Verrucomicrobia and Proteobacteria elevated in the MS group with the reduction of Bacteroidetes. At genus level, there was a significant difference between MS and MC groups, indicating that MS disrupts the balance of gut

microbiota. The main genera included Akkermansia, Alistipes, Barnesiella, Blautia, Corynebacterium, Paraprevotella, Robinsoniella, Saccharibacteria_genera_inc-ertaesedis, and Streptococcus. Compared with MC group, MS dramatically increased the abundance of Akk-

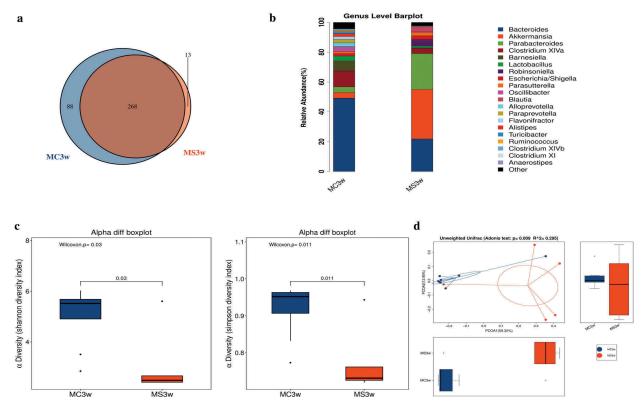


Figure 4. Maternal sucralose intake altered composition of gut microbiota in 3-week-old pups. Stool samples of 3-week-old offspring were collected for 16 S rRNA gene sequencing. Venn diagram was shown (a). Relative abundance of bacteria at the genus level between two groups (b). Alpha diversity including Shannon and Simpson diversity index (c). PCoA with Adonis test indicating Beta diversity was shown (d). MC, maternal control diet. MS, maternal sucralose intake. PCoA, principal coordinate analysis.MC: n = 8, MS: n = 5. *p < .05, **p < .05.

ermansia, Blautia, Corynebacterium, and Robinsoniella, meanwhile, Alistipes, Barnesiella, Paraprevotella,

Saccharibacteria_genera_incertae_sedis, and Streptococcus were depleted (Figure 4b).

We used the Shannon and Simpson diversity index to evaluate microbial alpha diversity of 3-week-old pups. MS significantly decreased alpha diversity (Figure 4c). Next, we used principal coordinate analysis (PCoA) with Adonis test on unweighted UniFrac distances to calculate the differences among the samples of two groups. The results revealed that MS had a significant influence on the microbial composition in PCOA of 3-week-old pups (Figure 4d).

Finally, we studied the differentially abundant bacteria at the phylum, class, order, family, and genus level between the two groups by LDA Effect Size analysis (Figure 5a). And Heatmap in each mouse was also presented (Figure 5b). Specifically, the relative abundance of anti-inflammation associated microbiota *Alistipes*¹⁷

were lower in MS group. Parabacteroides, 18 which can modulate host metabolism and alleviate obesity and metabolic dysfunctions were dramatically reduced in MS group. In contrast, Blautia¹⁹ which are highly related to obesity and metabolic disorders significantly increased in MS group. Moreover, the relative abundance of Akkermansia of Verrucomicrobia, a mucindegrading bacterium, 20 increased in MS group, possibly due to the disrupted mucosal barrier function and increased inflammation in the 3-week-old progeny. Consistent with increased intestinal low-grade inflammation in MS group, Escherichia/Shigella and Anaerostipes which were shown to be more abundant in inflammatory bowel disease and metabolic disgroup. 21,22 order also increased in MS Streptococcus and Ruminococcus, which were reduced in MS group, were shown to be negatively associated with inflammation in previous studies. 23,24 Intriguingly, butyrate-producing Saccharibacteria_genera_incertae_sedis,

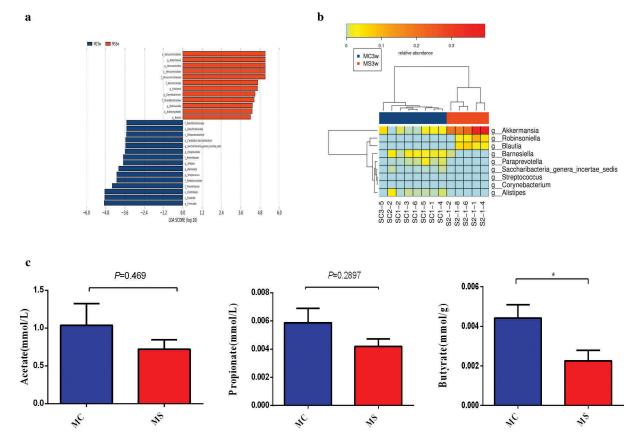


Figure 5. Maternal sucralose intake altered the key bacteria and SCFAs production in 3-week-old pups. Differentially abundant bacteria at the phylum, class, order, family, and genus level between two groups by LDA EffectSize analysis was shown (a) and Heatmap in each mouse (b). The levels of SCFAs including butyrate, acetate, and propionate in cecal contents were determined by HPLC analysis method (c). MC, maternal control diet. MS, maternal sucralose intake. SCFAs, short-chain fatty acids. MC: n = 8, MS: n = 5. *p < .05.

Prevotellaceae, and Clostridium XlVa dramatically decreased in the MS group of 3-week-old offspring. Overall, these results suggest that during pregnancy and lactation MS can perturb the gut microbiome in the offspring.

MS reduced short-chain fatty acids (SCFAs) in cecal contents of 3-week-old offspring mice

Bacterial fermentation of carbohydrates can produce intestinal SCFAs such as acetate, propionate, and butyrate. It has been reported that the gut barrier function was positively related to the concentrations of intestinal SCFAs. 25,26 In the present study, we found a significantly lower level of butyrate production in MS group than that in MC group, and the trend of acetate and propionate production in MS group also went downward (Figure 5c).

These findings along with the microbiota change observed above indicate that decreased SCFAs-producing bacteria and drawdown of butyrate production may play a part in the inhibition of intestinal development and the destruction of the intestinal barrier driven by MS intake.

MS increased the expression of GPR43 in 3-weekold offspring mice

It has been reported that butyrate activates the G-protein-coupled receptors (GPCRs) including GPR41, GPR43, and GPR109A, which are expressed on intestinal epithelial cells and adipocytes. Activation of these GPCRs appears to alleviate the progression of NAFLD via multiple mechanisms.²⁷

We evaluated the expression of GPR41, GPR43, and GPR109A in the colon in 3-week-old offspring by immunohistochemistry. We found that GPR41, GPR43, and GPR109A were all expressed in colon

tissues both in MS and MC groups. However, the expression of GPR43 in the colon in MS group significantly decreased than that in MC group, accompanied by the reduction of the butyrate-producing bacteria and butyrate (Figure 8a). The Real-time-PCR data also indicated that the relative expression of GPR43 decreased in the colon tissues of 3-week-old offspring than that in MC group (Figure 8b).

Clostridium butyricum decreased inflammatory cytokines production through GPR43 activation in vitro

Compared with the control, the levels of GPR43 mRNA were up-regulated 2.2-fold and 2.8-fold in HCT116 cells stimulated by sodium butyrate and Clostridium butyricum supernatant, respectively. The levels of GPR109A mRNA were upregulated by 2.9-fold and 2.1-fold. However, no significant change was observed in GPR41 mRNA expression. Meanwhile, Clostridium butyricum supernatant and sodium butyrate also caused 26.3% and 36.1% downregulation of the expression of TNFa, respectively. Furthermore, we also studied anti-inflammatory whether function Clostridium butyricum relayed on the GPR43 through siRNA-mediated GPR43 gene silencing. We found that the silencing of GPR43 blocked the Clostridium butyricum-mediated downregulation of TNF-α expression. The similar results were also presented in HCT8 cells (Figure 8c-g). These results indicated that the Clostridium butyricum has an anti-inflammatory effect through GPR43 activation.

MS exacerbated HFD-induced hepatic steatosis in adult mice

Accumulating studies have demonstrated that gut microbial imbalance and chronic inflammation in early life are potentially associated with adverse outcomes in adulthood. Here we further investigated whether MS intake in early life could increase the risk of hepatic steatosis in adulthood.

In general, the liver color of MS group appeared light yellow while that of MC group was dark red (Figure 6a). The weight of the liver and abdominal adipose tissues in MS increased more remarkably

than that of the MC group (Figure 6b). Alanine aminotransferase and aspartate aminotransferase increased dramatically in MS group compared with MC group. Furthermore, MS group had significantly higher serum levels of total cholesterol (TC) and triglyceride (TG) compared with MC group (Figure 6c).

We assessed the hepatic histology in 3-weekold offspring by H&E staining. There was no significant macroscopic inflammation in the two groups (Figure S1A). However, the relative expression of TNF-α, IL-1β, and IL-6 significantly increased in MS group compared with MC group (Figure S1B). H&E staining confirmed prominent fatty liver in both two groups of 12-weeks old. More importantly, the steatosis in MS group was more serious than that in MC, with plenty infiltration of inflammatory cells and emergence of partial piecel necrosis. Consistent with this, NAFLD Activity Score (NAS) was also significantly higher in MS group than that in MC (Figure 6d). Additionally, the inflammatory cytokines in liver such as IL-6 and TNF-α also elevated in MS group compared with MC group (Figure 6e).

MS disturbed hepatic lipid metabolism in adult mice

The levels of several genes associated with lipid metabolism in liver were detected. The fatty acid synthesis genes such as *Fasn* and *Acaca* elevated in MS group compared with MC. *Cyp7a1*, the rate-limiting enzyme for bile acid synthesis was suppressed in MS group, and *shp*, also a key gene regulating bile acid synthesis in the liver, was highly upregulated (Figure 6f). These genes expression alteration suggested that MS disturbed the biosynthesis and metabolism of fatty acid and bile acids.³⁰

HFD exacerbated dysbiosis in adult mice

Our studies then focused on the gut microbiota change after 4 weeks HFD in MS and MC groups. A total of 336 OTUs in MS group and 285 OTUs in MC group were shown by Venn diagram, with 269 OTUs shared (Figure 7a). Compared with 4 weeks ago, the relative

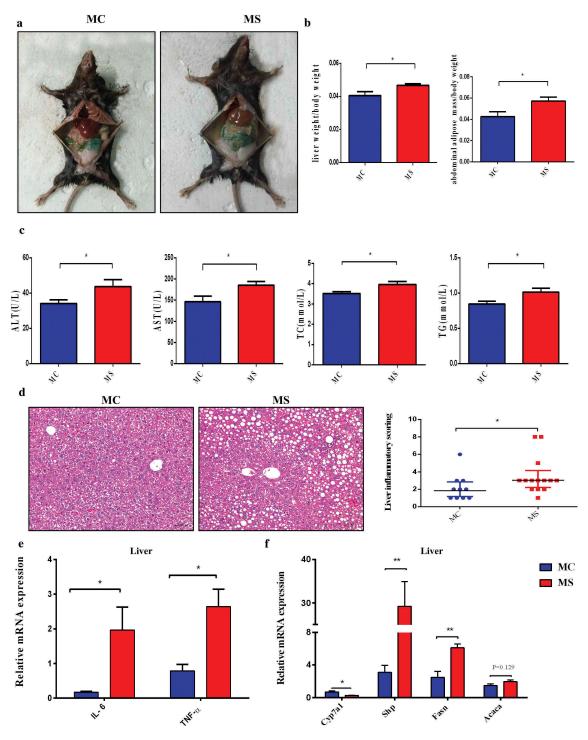


Figure 6. Maternal sucralose intake exacerbated high-fat diet-induced hepatic steatosis in 12-week-old offspring mice. The liver weight and abdominal adipose mass were measured at 12 weeks (a-b). Serum biochemical index including ALT, AST, TC, and TG was detected (c). Hepatic histology by H&E staining was shown, and liver inflammatory scoring was evaluated (d).Relative expression of IL-6 and TNF- α in the liver (e). Relative expression of lipid metabolism genes including *Cyp7a1*, *shp*, *Fasn*, and *Acaca* in liver was detected (f). MC, maternal control diet. MS, maternal sucralose intake. ALT, alanine aminotransferase. AST, aspartate aminotransferase. TC, total cholesterol. TG, triglyceride.MC: n = 10, MS: n = 14. Scale bar: 50 µm. *p < .05, **p < .01.

abundance of *Firmicutes* and *Proteobacteria* elevated while *Bacteroidetes* reduced in both two groups. At phylum level, even after

9 weeks HFD, the abundance of *Proteobacteria* decreased more significantly in MS group than in MC group (Figure 7b).

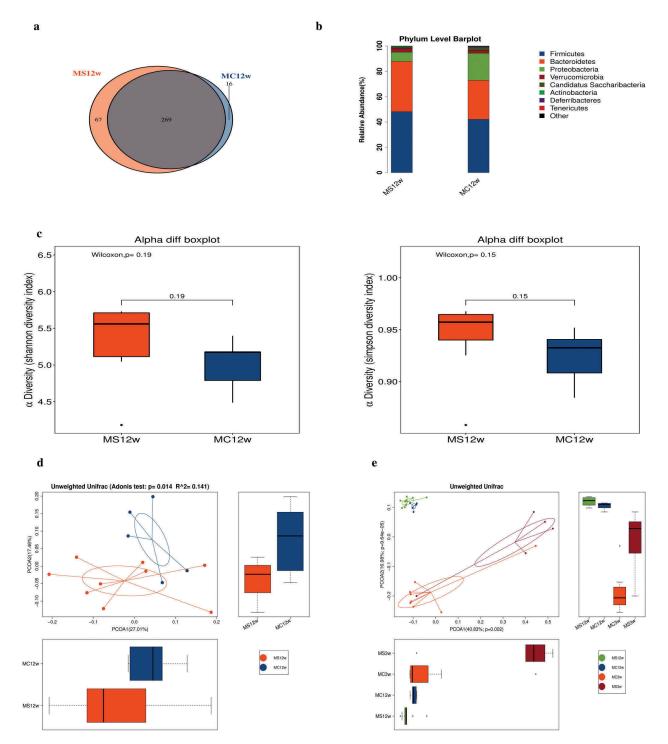


Figure 7. Maternal sucralose intake exacerbated gut dysbiosis in 12-week-old offspring mice. Venn diagram (a). Relative abundance of bacterial genera in two groups (b). Alpha diversity including Shannon and Simpson diversity index (c). PCoA with Adonis test indicating Beta diversity was shown (d). PCoA at 3 and 12 weeks offspring based on unweighted UniFrac distances (e). MC, maternal control diet. MS, maternal sucralose intake. PCoA, principal coordinate analysis. MC: n = 5, MS: n = 9. *p < .05, **p < .01.

There was no significant difference found between two groups by Shannon and Simpson diversity index which represent alpha diversity after 4 weeks HFD (Figure 7c). Referring to Beta diversity, Adonis test showed significant difference between the two groups (Figure 7d). Notably, PCoA at 3- and 12-week-old offspring based on unweighted UniFrac distances revealed that the HFD indeed partly

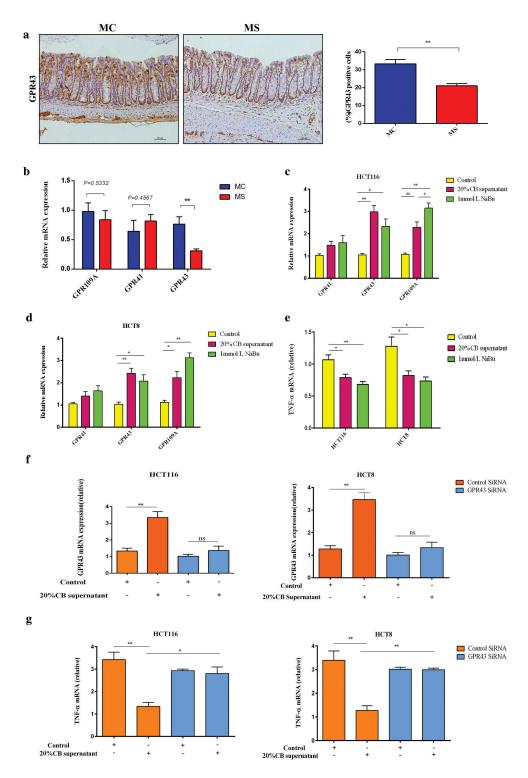


Figure 8. Clostridium butyricum decreased inflammatory cytokines production through activating GPR43. Immunohistochemical staining of GPR43 in the colonic tissues of 3-week-old offspring (a). The relative expression of GPR41, GPR43, and GPR109A in colonic tissues of 3-week-old offspring was presented by real-time PCR (b). The relative mRNA expression of GPR41, GPR43, and GPR109A when stimulating with Clostridium butyricum and sodium butyrate in HCT116 cells were presented (c). The relative mRNA expression of GPR41, GPR43, and GPR109A when stimulating with Clostridium butyricum and sodium butyrate in HCT8 cells were presented (d). The relative mRNA expression of TNF-a when stimulating with Clostridium butyricum and sodium butyrate in HCT116 and HCT8 cells was shown (e). The expression of GPR43 in Control SiRNA and GPR43 siRNA groups of HCT116 and HCT8 cells (f). Expression of TNF-α in GPR43-knockdown HCT116 and HCT8 cells relative to the control (D). MC, maternal control diet. MS, maternal sucralose intake. CB, Clostridium butyricum. NaBu, sodium butyrate. MC: n=7, MS: n=5. Scale bar: 50 μ m. *P<.05, **P<.01, ***P<.001.

diluted the difference of gut microbiome driven by MS (Figure 7e). Together, our results revealed that HFD seriously exacerbated the dysbiosis of offspring gut microbiota. However, even after HFD, the gut microbiota of MS group was still partly different from MC group after 4 weeks HFD.

Discussion

NAFLD has become the most common liver disease worldwide but the pathogenesis has not been fully illuminated. A growing body of evidence supports the concept that maternal diet is associated with NAFLD through altering microbiota.31,32 It is reported that sucralose can alter the composition of the gut microbiome and affect host health.^{8,9} For example, maternal exposure to sucralose and acesulfame-K during pregnancy and lactation impacts hepatic metabolism and gut microbiome of offspring pre- and postweaning.¹² However, the long-term results of offspring in adulthood with MS consumption remain unknown. The main finding of our study was that MS supplement during pregnancy and lactation can inhibit intestinal development, disrupt gut barrier integrity, alter intestinal microbial composition, and increase inflammatory cytokines formation in offspring mice and finally aggravate HFD-induced hepatic steatosis in adulthood. In addition, MS intake reduces the butyrateproducing bacteria and butyrate production of the offspring, impairs gut barrier and then leads to low-grade inflammation through GRP43. This is the first experimental evidence in support of the hypothesis that MS in early life may increase the susceptibility to hepatic steatosis of offspring in adulthood. More importantly, the potential mechanism may lie in that MS induces gut dysbiosis including decreasing butyrate-producing bacteria and butyrate production and alters the intestinal barrier function which supports the concept of "gut-liver axis."

It is widely believed that maternal diet is associated with progeny's body weight gain and obesity. 33,34 However, the effects of sucralose on the weight gain were not fully consistent in previous studies. Abou-Donia reported that administration of sucralose approximately 1.1 to 11 mg/kg BW/day from the 4th week after birth for 12 weeks

significantly increased body weight in rats.³⁵ While Takashi Uebanso showed that sucralose supplement approximately 14.2 mg/kg BW/day for 8 weeks did not change the body weight of mice.⁷ Chih-Wei Liu et al. also revealed that no significant body weight change was observed after sucralose intake with 0.1 mg/ml from 8 weeks old for 6 months. One of the main reasons of the differences may lie in the time and amount of sucralose intake. In our study, pups in MS group showed more body weight gain at weaning and after HFD. Interestingly, similar results were found in previous studies when maternal HFD was fed in the perinatal period. 4,36 Three-weekold is the key point for gut barrier formation and intestinal microbiota colonization, and microbes in early-life play a key role in body weight gain. 13,37 We may presume the effect of MS on offspring body weight through its impact on gut barrier and gut microbiota.

Undoubtedly, maternal nutrients are of the essence to the progeny's intestinal development. 38–40 Our previous report has already shown that probiotics supplementation in early life can promote intestinal proliferation and differentiation. In contrast, the effect of early sucralose consumption on the offspring intestinal development has not been investigated before. In our study, MS negatively impacts the intestinal development of offspring in early life. It is in accord with the hypothesis that nutrients are thought to regulate initial colonization of perinatal microbiota, and gut dysbiosis can change biological process in the intestinal tract and throughout the body. 29

The gut microbiota is a dynamic community, and maintaining a proper balance of it is vital for the host health. 42 Transmission of the microbiome from mother to offspring indeed exists and maternal gut microbiota is proved to be more persistent in the infant's gut and ecologically better adapted than those acquired from other sources.⁴³ Several studies have demonstrated that sucralose could alter the microbiota. 5-7,9,44 In our present study, maternal sucralose significantly altered composition and diversity of gut microbiota in 3-week-old offspring mice. It is consistent with the notion that the gut microbiota colonization initiates already prenatally by placenta and amniotic fluid, and continues by breast milk after birth. 45 In the report of Takashi Uebanso, sucralose intake from

weaning reduced the relative amount Clostridium cluster XIVa in feces of the mice. Our results showed that more abundant genera were involved in offspring mice with MS supplement, which supported the notion that the age of initiating intervention is a critical factor which influences the gut microbiota.⁴⁶ It is noteworthy that the gut microbiota in MS group was still partly different from MC group after 4 weeks HFD, which indicates that the impact of dysbiosis induced by MS is far-reaching.

In the present study, MS can promote the production of pro-inflammatory mediators and trigger low-grade intestinal inflammation in offspring at 3 weeks of age, which supports the widely accepted notion that inflammation is one of the most common effects associated with gut microbiome dysbiosis. 47,48 On one hand, dysbiosis can influence the maturation of inducible regulatory T cells (iTregs) induction, which may be responsible for the decreased secretion of anti-inflammatory cytokines. 49,50 On the other hand, among the more abundant genera in MS group, several genera were found to be associated with inflammation. Moreover, decreased abundance of SCFA-producing bacteria was also associated with the reduction of iTregs and activation of proinflammatory cells. 51-53

As one of the most important aspects of gut barrier function, further damage to the integrity of the gut barrier also goes along with dysbiosis and intestinal low-grade inflammation.¹⁵ In this study, we observed a reduction of MUC2 positive cells and goblet cells and in the colon, together with reduced tight junction proteins. These findings suggest that MS significantly disrupts the gut barrier integrity of offspring at 3 weeks. Meanwhile, the increased abundance of Akkermansia may be partly responsible for the decreased secretion of mucin in goblet cells.⁵⁴

Additionally, our results revealed that MS reduced the butyrate-producing bacteria in 3-week-old offspring, which was consistent with their cecal butyrate decline. Butyrate-producing bacteria and butyrate have positive effects on the host's health in several aspects. Butyrate has been reported to inhibit histone deacetylase activity, regulate macrophage function, as well as remodel regulatory T cell expansion in mice. 55,56 Butyrate also fine-tunes the

immune system and metabolic homeostasis in early life, potentially setting the stage for long-term metabolic and immune health.^{57–59} In addition, increasing evidence has shown that butyrate also plays a critical role in the development of NAFLD.²⁷ It has been reported that butyrate enhances the intestinal barrier function by facilitating tight junction assembly via activation of AMP-activated protein kinase (AMPK) and attenuates HFD-induced steatohepatitis. 60,61 Moreover, Hitoshi Endo et al. showed that treatment with a butyrate-producing probiotic, MIYAIRI 588, significantly reduced hepatic lipid deposition and improved the triglyceride content, insulin resistance, serum endotoxin levels, and hepatic inflammatory indexes.⁶² In our present study, we also found that the expression of proinflammatory factors such as TNF-asignificantly increased in liver of 3-week offspring in MS group. Besides, they found that MIY AIRI 588 also improved the expression of the intestinal tight junction proteins such as ZO-1 and Occludin. And we may consider the down-regulated expression of tight junction proteins in our present study was partly due to the reduced butyrate-producing bacteria and butyrate. It has been reported that butyrate activates the GPCRs including GPR41, GPR43, and GPR109A, which are expressed on intestinal epithelial cells and adipocytes. Activation of these GPCRs appears to alleviate the progression of NAFLD via multiple mechanisms.²⁷ In our present study, we found a significantly decreased expression of GPR43 in colon in MS group. Moreover, in in vitro study, we found that one of the butyrate-producing bacteria inhibited pro-inflammatory factors via activating GPR43. Thus, we consider that sucralose intake could reduce the butyrate-producing bacteria and butyrate production of the offspring, impair gut barrier and then lead to low-grade inflammation through GRP43.

According to the conception of "gut-liver axis," low-grade inflammation and impaired intestinal barrier play a critical role in promoting the progression and possibly the initiation of NAFLD. 63,64 The increased pro-inflammation mediators and detrimental metabolites in the gut such as endotoxin can translocate into portal system through the impaired gut barrier, leading to multiple biologic damage to the liver. In the study of Stephanie Olivier-Van Stichelen et al, 12 they investigated how the progeny's hepatic metabolic changed till 19 days after birth with maternal non-nutritive sweeteners intake. The offspring were breastfed during the experiment without other kinds of diet. They found no macroscopic damage to the liver, but both carbohydrate and lipid metabolism were found to be altered in the non-nutritive sweetener pups. In our present study, we also found MS did not induce obvious macroscopic hepatic inflammation in offspring at weaning, although the pro-inflammatory cytokines significantly increased. And we found that MS finally exacerbated HFD-induced hepatic steatosis in adult mice. These data strongly support the conception of "gut-liver axis."

MS intake during pregnancy and lactation disturbs the microbiome of mothers. Transmission of the microbiome from mother to offspring indeed exists, thus gut dysbiosis can be found in the neonatal stage of the offspring, accompanied by the reduction of butyrate-producing bacteria and butyrate production. Butyrate has an effect of suppressing inflammation and improving gut barrier function through activating GRP43. With the reduction of butyrate, pro-inflammatory factors in the gut lumen are highly increased, and the junction is dramatically impaired. tight According to the concept of "gut-liver axis," the endotoxin and the other detrimental microbial metabolites may rush into portal vein through the impaired gut barrier and upregulate proinflammatory cytokines production in the liver. The constant low-grade inflammation further promotes hepatic steatosis in adulthood in MS group.

The potential mechanisms of how MS intake alters gut microbiota of offspring and exacerbates hepatic steatosis in adulthood are presented in Figure 9.

In summary, this study first demonstrates that MS intake can change the composition and diversity of gut microbiome, reduce buty-rate-producing bacteria and butyrate, interfere intestinal development, induce low-grade inflammation as well as disrupt gut barrier function in 3-week-old offspring. Additionally, the microbial analysis in adulthood reveals that the impact of dysbiosis induced by MS is long-lasting, which may contribute to the exacerbation of the susceptibility of hepatic steatosis in

adulthood. And finally, MS exacerbated HFD-induced hepatic steatosis in adulthood. These data strongly support the conception of "gut-liver axis," and that MS intake may be a potential threat for NAFLD in adulthood. As sucralose is widely used around the world, our findings may remind the pregnant women that more caution should be given to excessive sucralose consumption.

Methods and materials

Animals and diet

Eight-weeks old female C57BL/6 mice were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences & Peking Union Medical College. After 2 weeks of acclimation in the mouse facility, they were a 12-h light/dark cycle exposed to a temperature and humidity-controlled specificpathogen-free environment. After mating with C57BL/6 males, all the pregnant female mice were randomly divided into two groups: On the basis of standard rodent chow (3.85 kcal/g; 10 E% fat, 20 E% protein, 70 E% carbohydrate; H10010, Research Diets, Peking, China), maternal control group mice were given distilled water; MS group mice were given sucralose solution of 0.1 mg/ml, approximately 5-15 mg/kg BW/day, and equal to upper limit of FDA acceptable daily intake.^{7,12} Throughout the period of gestation (3 weeks) and lactation (3 weeks), MS group mice consumed sucralose solution, whereas MC group mice consumed the same amount of distilled water as control on the basis of standard diet consistently.

Subsequently, pups were delivered through the vagina and the body weight was recorded weekly until 8 weeks. After weaning at 3 weeks of age, pups were separated from the dams, received standard rodent chow and regular drinking water *ad libitum* till 8 weeks. Finally, all the pups were treated with a HFD (HFD,5.24 kcal/g; 20 E% carbohydrate, 20 E% protein, 60 E% fat; H10060, Research Diets, Peking, China) for 4 weeks (Figure 1a). The research program was evaluated and approved by the Animal Ethical and Welfare Committee of Tianjin Medical University.

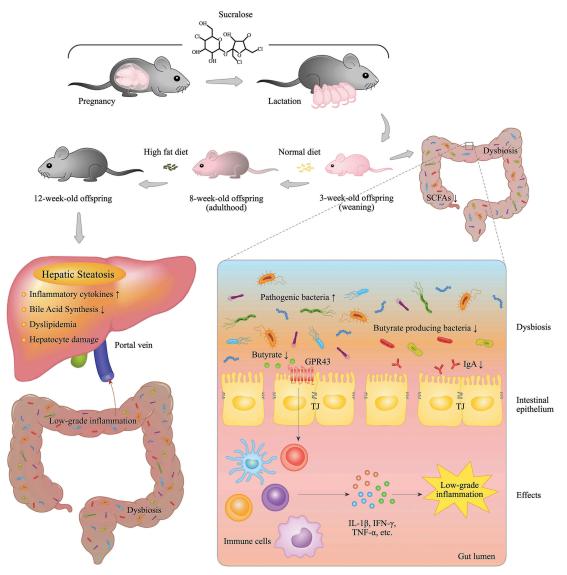


Figure 9. The potential mechanism of how maternal sucralose intake alters gut microbiota of offspring and exacerbates hepatic steatosis in adulthood. Maternal sucralose intake during pregnancy and lactation disturbs the microbiome of mothers. Transmission of the microbiome from mother to offspring indeed exists, thus gut dysbiosis can be found in the neonatal stage of the offspring, accompanied by the reduction of butyrate-producing bacteria and butyrate production. Butyrate has an effect of suppressing inflammation and improving gut barrier function through activating GRP43. With the reduction of butyrate, pro-inflammatory factors in the gut lumen are highly increased, and the tight junction is dramatically impaired. According to the concept of "gut-liver axis," the endotoxin and the other detrimental microbial metabolites may rush into portal vein through the impaired gut barrier and upregulate pro-inflammatory cytokines production in the liver. The constant low-grade inflammation further promotes hepatic steatosis in adulthood in MS group.

Blood measurements and tissue collection

Blood was drawn, centrifuged (3000 rpm, 8 min, 4°C) and serum was collected. Serum liver enzymes including ALT, AST, and lipid including TG and TC were determined by enzymatic colorimetric method.

Mice were sacrificed via CO2 inhalation at 3 and 12 weeks of age. Liver, small intestine, colonic tissues, cecal contents, and abdominal adipose

tissue were dissected immediately. The weight of liver and abdominal adipose was measured. Liver tissues and intestinal specimens were quickly stored in a refrigerator at -80°C for further extraction of RNA and protein. A part of liver and intestinal tissues were washed with ice-cold phosphate-buffered saline (PBS) solution and formalinfixed for further pathological evaluation. Before ending the experiment, we collected the fresh

stool samples and then froze in the liquid of nitrogen for gut microbiota analysis.

Histology, immunohistochemistry, and immunofluorescence

To evaluate the histology of liver, the paraffin sections of 4-µm thick were stained by hematoxylin-eosin (HE) in the 3-week-old and 12-week-old offspring. The general histological features, extent of steatosis, inflammation, and fibrosis were evaluated. NAS was used for scoring the severity of hepatic steatosis as previously detailed. 65,66

The paraffin sections of small intestine and colon were also stained with H&E. The histological inflammation of colon was evaluated by the degree of crypt damage. The intestinal development was evaluated by measuring villus/crypt length. We randomly chose 300 wellorientated villi/crypts and observed under light microscopy at 200× magnification for each mouse. The expression of Ki67 in small intestine and mucin 2 (MUC2), G-protein coupled receptor 43 (GPR43) in colon were evaluated by immunohistochemistry. Deparaffinized sections were incubated with primary antibodies rabbit monoclonal anti-Ki67 (ab16667, Abcam, Cambridge, MA, USA), rabbit anti-MUC2 (Santa Cruz Biotechnology, Inc), and GPR43 (ab118449, Abcam, Cambridge, MA, USA) overnight at 4°C. After cleaning with PBS, the secondary antibody (Santa Cruz Biotechnology, Inc.) labeled with horseradish peroxidase (HRP) was cultured for half an hour. Finally, 3,3⊠-diaminobenzidine was used for staining. The same pathologists observed them blindly with optical microscopy Germany).

The expression of zona occludens-1 (ZO-1) in colon and IgA in small intestine was assessed by immunofluorescence. Deparaffinized sections were first incubated at 4°C overnight with rabbit anti-ZO-1 antibody (Invitrogen Corporation, Carlsbad, CA) or anti-IgA antibody (Invitrogen Corporation, Carlsbad, CA), and then secondary incubation was processed for 1 h at room temperature with FITC-labeled anti-rabbit antibody (Cell Signaling

Technology). Finally, the nuclei were stained with DAPI (4,6-diamino-2-phenylindole, blue) and observed with fluorescence microscope (Leica, Germany).

PAS staining

Deparaffinized sections of colon were immersed for 10 min with 1% Periodic acid solution (Sigma-Aldrich), then stained with Schiff reagent (Sigma-Aldrich) for 40 min again. The PAS-stained sections were counterstained for another 2–5 min using Hematoxylin. During each step, washing buffer was an extensive PBS solution.

Table 1. Primer sequences used in real-time PCR.

Tubic 1. I fillici	sequences used in real-time PCh.
Primers	Sequence
mGAPDH	Forward 5'- GGAGAAACCTGCCAAGTATG -3'
	Reverse 5'- TGGGAGTTGCTGTTGAAGTC -3'
mIL-6	Forward 5'- CCAGTTGCCTTCTTGGGACT -3'
	Reverse 5'- GGTCTGTTGGGAGTGGTATCC -3'
mINF-γ	Forward 5'- GCATCTTGGCTTTGCAGCT -3'
	Reverse 5'- CCTTTTTCGCCTTGCTGTTG -3'
mTNF-α	Forward 5'- GTTCTGTCTACTGAACTTCGGG -3'
	Reverse 5'- GAGGCTTGTCACTCGAATTTTG -3'
mIL-1β	Forward 5'- ACGGACCCCAAAAGATGAAG -3'
	Reverse 5'- TTCTCCACAGCCACAATGAG -3'
mMUC2	Forward 5'- TCGCCCAAGTCGACACTCA -3'
	Reverse 5'-GCAAATAGCCATAGTACAGTTACACAGC-3'
mZO-1	Forward 5'- GGGCCATCTCAACTCCTGTA -3'
	Reverse 5'- AGAAGGGCTGACGGGTAAAT -3'
mOccludin	Forward 5'- ACTATGCGGAAAGAGTTGACAG -3'
	Reverse 5'- GTCATCCACACTCAAGGTCAG -3'
mClaudin-1	Forward 5'- GAATTCTATGACCCCTTGACCC -3'
	Reverse 5'- TGGTGTTGGGTAAGAGGTTG -3'
mClaudin-3	Forward 5'- CCTGTGGATGAACTGCGTG -3'
	Reverse 5'- GTAGTCCTTGCGGTCGTAG -3'
mAcaca	Forward 5'- TCACGCCACCTTGTCAG -3'
	Reverse 5'- GGGGAGTCACAGAAGCAG -3'
mFasn	Forward 5'- TCGGTGTATCCTGCTGTCC -3'
	Reverse 5'- TTGGGCTTGTCCTGCTCT -3'
mShp	Forward 5'- GTGCCCAGCCATCAGAC -3'
	Reverse 5'- AGAGGATCGTGCCCTTCA -3'
mCyp7a1	Forward 5'- AGGCATTTGGACACAGAAG -3'
	Reverse 5'- ACAGATTGGAGGTTTTGCAT -3'
mGPR41	Forward 5'- GTGTAGTCTGTTGGTTCCTGG -3'
	Reverse 5'- TGTAGGTTGCATTTCCCCAG -3'
mGPR43	Forward 5'- AGGTTTGCTACTGATCCGC -3'
	Reverse 5'- GTACCCCTTCTGCTTGACTTC -3'
mGPR109A	Forward 5'- TTTCTCCAGCCCATCTTTCC -3'
	Reverse 5'- ATACTTCTGGTTGTGCTGGG -3'
hGPR41	Forward 5'- TGCGGTCAATGGGATCTG -3'
	Reverse 5'- CAAGCACAGAGACGTAAGAGG -3'
hGPR43	Forward 5'- TGTATGGAGTGATTGCAGCTC -3'
	Reverse 5'- TGGTTATCGGTGAAGTTCTCG -3'
hGPR109A	Forward 5'- CACTCTCAGCTTCACCTACATG -3'
	Reverse 5'- CTCACCTGTCATCTTCCTCTG -3'
hTNF-α	Forward5'- ACAGGGCAATGATCCCAAAG-3'
	Reverse 5'-CTCTTGATGGCAGAGAGGAGGTTGA-3'



Real-time PCR analysis

Total RNA of the liver, intestine, and cells were extracted with the RNeasy mini kit (Qiagen, Carlsbad, CA, USA). cDNA synthesis was perusing the **TIANScript** (TIANGEN, Inc. Beijing, China). And realtime-PCR analyses were performed described previously.⁶⁷ We chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control, and the mRNA levels were calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences of target genes are listed in Table 1.

SCFAs analysis

The concentrations of SCFAs (including acetate, propionate, and butyrate) in cecal contents were determined by high-performance liquid chromatography (HPLC) analysis method. Sample preparation and HPLC analysis were carried out according to the previous studies. 66,68,69 For data interpretation, Agilent Chem Station software was used.

Gut microbiota analysis

Stool samples of 3-week-old pups and 12-weekold mice in two groups were sent to the Real bio Genomics Institute (Shanghai, China) for 16 S rRNA gene sequencing. Each stool sample was extracted according to the manufacturer's instructions (QIAamp DNA Stool Mini Kit, Qiagen, Hilden, Germany). General primers (341 F: ACTCCTACGGGAGGCAGCAG, and 806 R: GGACTACHVGGGTWTCTAAT) were used to amplify 16 S rDNA, targeting 16 S genes V3-4 region. KAPA HiFi Hot start PCR kit was used for high fidelity PCR. Illumina TruSeq DNA PCR-Free Library Preparation Kit (Illumina, USA) was utilized to generate QIIME software package (Quantitative Insights Into) as well as sequencing libraries. Sequences were expressed as OTUs. Afterward, QIIME platform was used for further classification analysis on the basis of OTUs sequences. Diversity was also analyzed.

Cells and bacterial preparation

Clostridium butyricum was used as the representative of butyrate-producing bacteria because it was one of the most commonly used butyrateproducing bacteria in clinical studies and basic science. 70-74 Clostridium butyricum (ATCC 19398), which was kindly provided by China General Microbiological Culture Collection Center was incubated in brain heart infusion medium at 37°C in an anaerobic environment for 24 h. And then the bacteria were harvested with centrifugation (3000 $g \times 5$ min) and resuspended in sterile PBS at the density of 1×10^{10} CFU/ml. Then, we collected the supernatant, filtered it through aperture filters of 0.22-µm, and then diluted it with complete culture medium.

We obtained human colorectal cancer cell lines (HCT8 and HCT116) from American Type Culture Collection (ATCC, Manassas, VA, USA). HCT116 cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS); while HCT8 cells were grown in Roswell Park Memorial Institute 1640 medium with 10% FBS. Both the cells were maintained in a humidified atmosphere with 95% air and 5% carbon dioxide at 37°C.

Treatment of HCT116 and HCT8 cells with Clostridium butyricum

HCT116 and HCT8 cells were stimulated with 1 mmol/L sodium butyrate or Clostridium butyricum supernatant at a concentration of 20% for 24 h for further analysis. The treating concentration and time were selected based on our previous research.⁷⁴ The treatment lasted for 24 h and then the cells were collected for real-time PCR analysis. The relative expression of GPR43 and TNF-α was detected.

Gene silencing of GPR43 using siRNA

We applied small interfering RNA technology at the aim of knocking down GPR43 in the HCT8 and HCT116 cells as described in previous experiment. The targeted sequences of the selected region to be targeted by siRNA for GPR43 were the sense strand, 5'-GGCCUGGGUUAUGUCCUUUTT-3', the antisense strand, 5'-AAAGGACAUAACCCAGGCCTT -3' (GenePharma, Shanghai, China) and negative control siRNA (nontargeting siRNA)were sense strand, 5'-UUCUCCGAACGUGUCACGUTT-3', antisense strand, 5'-ACGUGACACGUUCGGAGA ATT-3'. HCT116 and HCT8 Cells were seeded into six-well plates and transfected with GPR43 siRNA of 2 μg or control siRNA with Lipofectamine 2000 (Invitrogen) in Opti-MEM. The process followed the manufacturer's instructions, respectively. After 6 h, culture medium was replaced and 20% *Clostridium butyricum* supernatant or 1 mmol/L sodium butyrate was added. The treatment lasted for 24 h and then the cells were collected for real-time PCR analysis. The relative expression of GPR43 and TNF-α was detected.

Data analysis

Data Statistical analysis and graph design were implemented using SPSS 21.0 (SPSS, Chicago, IL, USA) and Prism (GraphPad Software, La Jolla, CA, USA). Data were expressed as the mean \pm SEM. Rank sum test or non-paired t-test was used for the comparison of the two groups. One-way ANOVA was applied for comparison of multiple groups. A p value less than 0.05 was defined as statistically significant.

Author Contributions

XD, ZXG, DFC, LL, XLS, TYL, GJ, YL, YL, and AA performed experiments and analyzed data. XD, ZXG, and DFC drafted the manuscript and created figures; XD and HLC conceived the project; CY, BMW, and HLC directed the research and made the critical revision. All authors identified the manuscript and finally approved the article.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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